(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
8 March 2001 (08.03.2001)

PCT

(10) International Publication Number
WO 01/15737 A2

(51) International Patent Classification[7]: A61K 47/48

(21) International Application Number: PCT/GB00/03306

(22) International Filing Date: 25 August 2000 (25.08.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
9920397.8 27 August 1999 (27.08.1999) GB
0012083.2 18 May 2000 (18.05.2000) GB

(71) Applicant *(for all designated States except US)*: MEDICAL RESEARCH COUNCIL [GB/GB]; 20 Park Crescent, London W1N 4AL (GB).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: GAIT, Michael, John [GB/GB]; 16 Forest Road, Cambridge CB1 9JB (GB). STETSENKO, Dmitry [RU/GB]; Upper Flat, 25 Fulbourn Road, Cambridge CB1 4JL (GB).

(74) Agents: HOWARD, Paul, Nicholas et al.; Carpmaels & Ransford, 43 Bloomsbury Square, London WC1A 2RA (GB).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:

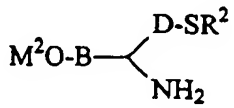
— *Without international search report and to be republished upon receipt of that report.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/15737 A2

(54) Title: METHOD FOR COUPLING MOLECULES

$$M^1\text{-NH-CO-A-CO-SR}^1 \qquad (I)$$



(II)

(57) Abstract: A method of linking a first molecule M^1-NH_2 with a second molecule M^2-OH comprising reaction of a compound of the formula (I): M^1-NH-CO-A-CO-SR^1 wherein M^1 is the residue of a molecule bearing an amino group, A is an alkylene or arylene group, R^1 is alkyl or aryl, with a compound of formula (II), M^2 is the residue of a molecule bearing a hydroxy group wherein B is a linker, D is C_{1-4} alkylene group or C_{3-12} arylene group, R^2 is hydrogen or a thiol protecting group. In addition, this invention relates to conjugate products of the coupling reaction, reagents for modifying M^1-NH_2 and M^2-OH, and kits comprising these reagents.

METHOD FOR COUPLING MOLECULES

The present invention relates to a method for coupling molecules such as peptides and oligonucleotides, as well as synthetic intermediates and coupling agents therefor.

Conjugates of, for instance, peptides and oligonucleotides have many potential applications. Recently, a number of peptides have been proposed as carriers of oligonucleotides or DNA into cells. These peptides may be covalently linked to oligonucleotides via an appropriate linker.

The conjugation of molecules such as peptides and oligonucleotides requires reliable coupling chemistry which should be widely applicable to conjugates that vary substantially in composition. To date, there have been very few methods available for synthesising conjugates which meet these criteria. Some strategies for coupling molecules such as peptides and oligonucleotides involve synthesis and/or attaching one or both of the coupling components onto a solid support. One of the advantages of carrying out coupling reactions on solid phase is the facile purification of the product. Consequently, the technology is amenable to automated synthesis.

Perhaps the most ambitious route to the synthesis of peptide-oligonucleotide conjugates has been the total stepwise solid-phase approach on a single solid support. However, such a route poses serious difficulties, both technically and chemically. Firstly, no automated machine exists which can handle a sufficiently wide range of reagents to carry out the sequential assembly of peptides and oligonucleotides. Secondly, there are severe problems of compatibility in the assembly chemistries, particularly in the choice of protecting groups and deprotection conditions. Such problems have limited this approach to relatively short peptides, the longest reported conjugate produced by such a method being a 13-mer D-peptide of IGF1 coupled to a phosphorothioate 15-mer (Basu *et al*, Tetrahedron Lett., 36 (1995) 4943).

A more general strategy for peptide-oligonucleotide conjugation is where peptide and oligonucleotide moieties are assembled separately on their own solid supports and are designed to carry a reactive functionality that is released upon full deprotection and cleavage from the support. Following purification, peptide and oligonucleotide parts are joined in aqueous or non-aqueous solution through the reactive functionalities. The methods currently available for coupling such functionalised peptides and oligonucleotides are somewhat limited. Amongst conjugation reactions in aqueous solution, Bongartz *et al* (Nucleic Acids Res., 22 (1994) 4681), Vives *et al* (Tetrahedron Lett., 38(1997) 1183) and Eritja *et al* (Tetrahedron Lett., 47(1991) 4113) have reported on coupling via formation of a disulfide bond. Eritja *et al* (Tetrahedron, 47 (1991) 4113) have reported coupling via reaction of a thiol oligonucleotide with a maleimide peptide. Harrison *et al* (Nucleic Acids Res., 26(1998) 3136) and Tung *et al* (Bioconj. Chem., 2 (1991) 464) have reported on coupling of a cysteine peptide with a maleimido oligonucleotide. Arar *et al* (Bioconj. Chem., 6 (1995) 573) reports on the coupling of a bromoacetyl peptide with a thiol-functionalised oligonucleotide. Soukchareun *et al* (Bioconj. Chem., 9 (1998) 466) have reported on coupling of a maleimido peptide with a 3'-cysteine functionalised oligonucleotide.

McMinn and Greenberg have reported the non-aqueous conjugation of a partially protected peptide via its carboxyl terminus or as an aryl isocyanate derivative to a 3'-amino functionalised oligonucleotide (McMinn and Greenberg, J. Amer. Chem. Soc., 120 (1998) 3289).

Some disadvantages of performing the conjugation reaction in aqueous or non-aqueous solution by published procedures are (a) the oligonucleotide often needs to be further functionalised after release from the support, (b) one or both of the coupling components often will need to be purified prior to conjugation, (c) a limitation in the peptide sequence (e.g. need for a cysteine), (d) inefficient conjugation due to secondary structure or poor solubility of the peptide or oligonucleotide components, and (e) difficulties in separation of coupling components from the product conjugate, especially when conjugation yields are poor.

The solid-phase fragment conjugation method involves the retention of one component on a solid support during conjugation whilst the other remains in solution. For example Grandas *et al* have reported the fragment conjugation of a tripeptide as an amido phosphoramidite derivative to a support bound hexanucleotide (Grandas *et al*, Nucleoside and Nucleotides, 14 (1995) 825). Peyrottes *et al* have reported the conjugation of several peptides via the carboxy termini to support bound oligonucleotides which have been 5'-amino functionalised (Peyrottes *et al*, Tetrahedron, 54 (1998) 12513; Nucleosides and Nucleotides, 18 (1999) 1443). This method has several advantages over the solution-phase strategies. One advantage is that an excess of one component (e.g. peptide) can be used to drive the reaction to completion and the excess unconjugated peptide can then be removed by simple filtration and washing. This simplifies and aids the product purification following conjugation.

A highly efficient and generally applicable conjugation reaction is essential to success for both solution phase and solid-phase fragment coupling. Conjugation yields on solid phase may be greatly affected by the nature of the coupling component loaded onto the support and the nature of the coupling component in solution. Both components need to be maintained in high solvation and this needs a method of conjugation that allows the solvation conditions to be varied as appropriate (for example aqueous, aqueous-organic mixture, aqueous in the presence of denaturing agents etc).

A recent method used for the synthesis of large peptides and small proteins involves "native ligation" of two largely unprotected peptide fragments (Dawson *et al*, Science, 266 (1994) 776; Wilken *et al*, Curr. Opinion Biotech., 9 (1998) 412), one containing a C-terminal thioester and the other an N-terminal cysteine.

In the case of peptide-oligonucleotide conjugations, Bruick *et al* (Chem. & Biol., 3 (1996) 49) have reported the ligation of a 3'-amino oligonucleotide to a peptide thio-ester oligonucleotide aligned on a DNA template, but the synthesis of the various components is cumbersome and the method is restricted to 3'-peptide conjugated oligonucleotides and requires a DNA template.

Consequently, there is a need to develop methods for coupling molecules such as peptides and oligonucleotides which is applicable to a wide range of substrates.

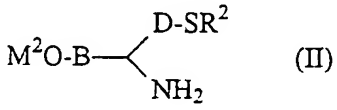
It is an object of this invention to provide a method of coupling molecules such as peptides and oligonucleotides using coupling chemistry which is both highly efficient and widely applicable. It is a further object of this invention to provide a method of coupling molecules such as peptides and oligonucleotides which can be used in solution and on solid phase and under a wide range solvent conditions that maintain solvation of the components. It is yet a further object of this invention to provide methods and reagents for modifying molecules such as peptides and oligonucleotides in order that they can be coupled together.

In accordance with the present invention there is provided a method of linking a first molecule M^1-NH_2 with a second molecule M^2-OH comprising reaction of a compound of formula (I)

$$M^1\text{-NH-CO-A-CO-SR}^1 \qquad \text{(I)}$$

wherein M^1 is the residue of a molecule bearing an amino group,
A is an alkylene or arylene group,
R^1 is alkyl or aryl,

with a compound of formula (II)



(II)

wherein M^2 is the residue of a molecule bearing a hydroxy group
B is a linker
D is a C_{1-4} alkylene group or C_{3-12} arylene group
R^2 is hydrogen or a thiol protecting group.

The method of the present invention is of general applicability to the coupling of molecules bearing an amino group to molecules bearing a hydroxy group. Accordingly, M^1 may be the residue of any molecule bearing an amino group and M^2 may be the residue of any molecule bearing a hydroxy group. More particularly, M^1 and M^2 may each independently comprise a peptide or oligonucleotide residue. Preferably, M^1 comprises a peptide residue. Preferably, M^2 comprises an oligonucleotide residue.

As used herein the term "peptide" refers to a molecule of up to 500 amino acid or peptoid (peptide-like) units. The amino acids may be naturally occurring, modified or synthetic amino acids. The peptide may be fully protected, partially protected or unprotected.

As used herein the term "oligonucleotide" refers to a molecule of up to 500 nucleotide units. The nucleotide units may be naturally occurring, modified or synthetic nucleotides or peptide nucleic acids (PNAs). The oligonucleotide may contain one or more chemically modified phosphate residue(s) (e.g. thiophosphate and the like) and non-phosphorus backbone substitutions (e.g. carboxamide and the like). The oligonucleotide may be fully protected, partially protected or fully unprotected.

As used herein, the term "alkyl" means a branched or unbranched, cyclic or acyclic, saturated or unsaturated (e.g. alkenyl or alkynyl) hydrocarbyl radical. Where acyclic, the alkyl group is preferably a C_1 to C_{18}, more preferably C_1 to C_{10}, more preferably C_1 to C_4 chain. Where cyclic, the alkyl group is preferably a C_3 to C_{12}, more preferably C_5 to C_{10} and more preferably C_5, C_6 or C_7 ring. The alkyl chain or ring may include (i.e. be optionally interrupted with and/or terminate with) one or more heteroatoms, such as oxygen, sulfur or nitrogen.

As used herein the term "alkylene" means a branched or unbranched, cyclic or acylic, saturated or unsaturated divalent hydrocarbyl radical. Where acyclic the alkylene group is preferably a C_1 to C_{18}, more preferably C_1 to C_{10}, more preferably C_1 to C_4 chain. Where cyclic, the alkylene group is preferably a C_3 to C_{12}, more preferably C_5 to C_{10}, more

preferably C_5, C_6 or C_7 ring. The alkylene chain or ring may include (i.e. be interrupted and/or terminate with) one or more heteroatoms such as oxygen, sulfur or nitrogen.

As used herein, the term "aryl" means a C_3 to C_{26}, preferably C_3 to C_{12}, aromatic group, such as phenyl or naphthyl, or a heteroaromatic group containing one or more, preferably one, heteroatom, such as pyridyl, pyrrolyl, furanyl, thienyl.

As used herein the term "arylene" means a divalent hydrocarbyl radical comprising a C_3 to C_{26}, preferably C_3 to C_{12}, aromatic group (such as o-, m- or p-phenylene) or heteroaromatic group containing one or more, preferably one, heteroatom (such as a pyridine-2,3-diyl group).

The alkyl, aryl, alkylene and arylene groups may be further substituted or unsubstituted. For example, a C_1 (methyl) group may be further substituted with a phenyl group to give a benzyl group. Substituents may include:

carbon containing groups such as

alkyl, aryl,

aralkyl (e.g. substituted and unsubstituted phenyl, substituted and unsubstituted benzyl);

halogen atoms and halogen containing groups such as

haloalkyl (e.g. trifluoromethyl);

oxygen containing groups such as

alcohols (e.g. hydroxy, hydroxyalkyl, aryl(hydroxy)alkyl),

ethers (e.g. alkoxy, alkoxyalkyl, aryloxyalkyl),

aldehydes (e.g. carboxaldehyde),

ketones (e.g. alkylcarbonyl, alkylcarbonylalkyl, arylcarbonyl, arylalkylcarbonyl, arylcarbonylalkyl)

acids (e.g. carboxy, carboxyalkyl),

acid derivatives such as esters

(e.g. alkoxycarbonyl, alkoxycarbonylalkyl, alkycarbonylyoxy, alkycarbonylyoxyalkyl)

and amides

(e.g. aminocarbonyl, mono- or dialkylaminocarbonyl, aminocarbonylalkyl, mono- or dialkylaminocarbonylalkyl, arylaminocarbonyl);

and carbamates

(eg. alkoxycarbonylamino, aryloxycarbonylamino, aminocarbonyloxy, mono- or dialkylaminocarbonyloxy, arylaminocarbonyloxy),

and ureas

(eg. mono- or dialkylaminocarbonylamino or arylaminocarbonylamino);

nitrogen containing groups such as

amines (e.g. amino, mono- or dialkylamino, aminoalkyl, mono- or dialkylaminoalkyl),

azides,

nitriles (e.g. cyano, cyanoalkyl),

nitro;

sulfur containing groups such as

thiols, thioethers, sulfoxides, and sulfones

(e.g. alkylthio, alkylsulfinyl, alkylsulfonyl, alkylthioalkyl, alkylsulfinylalkyl, alkylsulfonylalkyl, arylthio, arylsulfinyl, arylsulfonyl, arylthioalkyl, arylsulfinylalkyl, arylsulfonylalkyl);

and heterocyclic groups containing one or more, preferably one, heteroatom,

(e.g. thienyl, furanyl, pyrrolyl, imidazolyl, pyrazolyl, thiazolyl, isothiazolyl, oxazolyl, oxadiazolyl, thiadiazolyl, pyrrolidinyl, pyrrolinyl, imidazolidinyl, imidazolinyl, pyrazolidinyl, tetrahydrofuranyl, pyranyl, pyronyl, pyridyl, pyrazinyl, pyridazinyl, piperidyl, piperazinyl, morpholinyl, thianaphthyl, benzofuranyl, isobenzofuranyl, indolyl, oxyindolyl,

isoindolyl, indazolyl, indolinyl, 7-azaindolyl, benzopyranyl, coumarinyl, isocoumarinyl, quinolinyl, isoquinolinyl, naphthridinyl, cinnolinyl, quinazolinyl, pyridopyridyl, benzoxazinyl, quinoxalinyl, chromenyl, chromanyl, isochromanyl, phthalazinyl and carbolinyl).

As used herein, the term "alkoxy" means alkyl-O- and "alkanoyl" means alkyl-CO. Alkyl substituent groups or alkyl-containing substituent groups may comprise one or more further substituents.

As used herein, the term "aryloxy" means aryl –O- and "aryloyl" means aryl –CO. Aryl substituent groups or aryl-containing substituent groups may comprise one or more further substituents.

As used herein, the term "halogen" means a fluorine, chlorine, bromine or iodine radical, preferably a fluorine or chlorine radical.

According to a further aspect of the present invention there is provided a chemical compound of formula (I). The compound of formula (I) is suitable for use in a method of coupling M^1-NH_2 with M^2-OH. Preferably, M^1 comprises a peptide residue. When M^1 is a peptide residue, the amino group in M^1-NH_2 may be an N-terminal amino group, an internal amino group or it may be an amino group introduced at the C-terminal end of the peptide. Preferably, the amino group is an N-terminal amino group.

One advantage of the preparation of N-terminal thioesters is that the thioester group is spaced away from the terminal amino acid. This is helpful in that the C-terminal thioesters of peptides containing sterically hindered amino acids at the C-terminus, such as threonine, isoleucine, valine or proline, are known to couple very slowly in native ligation reactions (Hackeng *et al*, Proc. Natl. Acad. Sci. USA, (96), 1999, 10068-10073). A second

advantage is that there is no possibility of peptide racemization during conjugation reactions.

The peptide residue may be attached to a solid support. When the peptide residue is attached to a solid support, the solid support may be attached to the C-terminus, internally, or the N-terminus of the peptide residue. Preferably, the solid support is attached via the C-terminus of the peptide residue.

The compounds of the present invention may be prepared, and the methods of the present invention carried out, in solution phase or on a solid support, preferably on a solid support.

As used herein, the term "solid support" may be a solid support of any suitable type which will be readily apparent to those skilled in the art. Some examples of the solid supports which may be used in the present invention are polystyrene based resin such as polystyrene-co-divinylbenzene resins, polyacrylamide based resin such as PEGA resin, polyethylene glycol grafted polystyrene resin such as Tentagel® resin, PEG-PS resin and NovaGel™ resin, polyethylene/polypropylene based support such as functionalised polyethylene/polypropylene pins and crowns, or silica based support such as controlled pore glass (CPG). Preferably, the solid support used for attachment to the peptide residue is PAL-PEG-PS or NovaGel™ resin.

In an alternative embodiment, M^1-NH_2 comprises a peptide residue modified by introduction of an amino group at its C-terminus (by, for example, reaction of the C-terminal carboxyl group with a diaminoalkylene linker, optionally linked to a solid support, generating peptide C-terminal aminoalkylamide upon deprotection (see, for example, Breipohl *et al*, Tetrahedron Lett., 28 (1987) 5647).

In a further alternative embodiment, M^1-NH_2 may comprise an oligonucleotide residue which has been modified to incorporate an amino function. For example, an amino group may be introduced via an alkylene or arylene linker and phosphate or other group bonded via an oligonucleotide hydroxyl function (such as the 5^1 or 3^1 hydroxyl). Additionally, the

oligonucleotide may be linked to a solid support. Such an approach is exemplified in Nelson *et al*, Nucl. Acids Res., 17 (1989) 7179; Wachter *et al*, Nucl. Acids Res., 14 (1986) 7985; Agrawal *et al*, Nucl. Acids Res., 14 (1986) 6227. Additionally, the oligonucleotide may be linked to a solid support featuring built-in amino group liberated upon deprotection (Nelson *et al*, Nucl. Acids Res., 17 (1989) 7187; Nelson *et al*, Nucl. Acids Res., 20 (1992) 6253).

In the present invention A comprises an alkylene or arylene group as defined above. Preferably A comprises a C_{1-18} alkylene group, more preferably a C_2-C_4 alkylene group, more preferably an ethylene or n-propylene group.

R^1 may comprise an alkyl or aryl group as defined above. Preferably, R^1 comprises a C_{1-18} alkyl or C_{3-10} aryl group. More preferably, the compounds of formula (I) are selected from compounds in which R^1 is t-butyl, benzyl, substituted benzyl, phenyl, substituted phenyl, 2-pyridyl, 4-pyridyl cyanomethyl, carboxamidomethyl, 2-carboxamidoethyl or trifluoroethyl. Most preferably R^1 is benzyl.

It will be readily apparent to the skilled person that the reactivity of the thioester can be modified by varying R^1. For instance, the compound where R^1 is phenyl will be generally more reactive than the compound where R^1 is benzyl. Furthermore, the thioester of compound (I) may be transesterified *in situ* during the coupling reaction with the molecule of formula (II). The thioester may be transesterified by, for instance, thiophenol dissolved in the solvent.

In an alternative embodiment, R^1 further comprises a solid support. It will be appreciated that provision of a solid support permits solid phase synthesis or modification of the molecule of formula (I) by, for example, chain elongation of the peptide residue M^1 by conventional solid phase peptide synthesis techniques.

According to a further aspect of the present invention there is provided a chemical compound of formula (II). The compound of formula (II) is suitable for use in a method of coupling M^1-NH_2 with M^2-OH.

Preferably M^2 comprises an oligonucleotide residue. When M^2 is an oligonucleotide residue, the hydroxy group in M^2-OH may be a 5'-terminal hydroxy group, a 3'-terminal hydroxy group or another suitable internal hydroxy group. Preferably the oligonucleotide is bonded via its 5'-OH terminus.

The oligonucleotide residue may be attached to a solid support, as hereinabove defined. When the oligonucleotide residue is attached to a solid support, it may be attached via its 3'-OH terminus, internally or via its 5'-OH terminus. Preferably, when the oligonucleotide residue is part of molecule (II), it is attached via its 3'-OH terminus to a solid support such as controlled pore glass or polyoxyethylene-polystyrene co-polymer.

The group B may comprise any suitable linker. Preferably, group B comprises a group of the formula:-

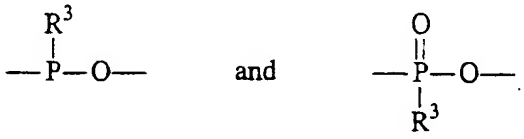
-X-J-

wherein J is an alkylene or arylene group and X is the residue of a functional group capable of reacting with a hydroxy group.

Preferably, J is a C_{1-18} alkylene or C_{3-12} arylene group, J may be functionalised, preferred functionalisation including carboxamido, urethane or sulfonamido groups. Preferably, J comprises a moiety derived from trans-4-aminocyclohexanol or 4-hydroxypiperidine.

X may be the residue of any suitable group capable of reacting with the hydroxyl function present M^2-OH. Preferably, X is a phosphate (including phosphoramidate), thiophosphate (including thiophosphoramidate), phosphonate or phosphite (including phosphoramidite, thiophosphite and thiophosphoramidite) residue. In case of hydrogen phosphonate and phosphite residue, it may be optionally oxidized, e.g. by aqueous iodine solution, t-

butylhydroperoxide and the like (Letsinger *et al*, J. Am. Chem. Soc., 97 (1975) 3278), sulfurized, e.g. using molecular sulfur solution or any suitable thionating reagent (Stec *et al*, J. Am. Chem. Soc., 106 (1984) 6077), or aminated, e.g. by amine – carbon tetrachloride solution (Froehler, Tet. Lett., 27 (1986) 5575). More preferably X comprises a group selected from



wherein R^3 is selected from hydroxy, oxy anion and salts thereof, alkyl alkoxy, aryloxy, thiol, thioxy anion and salts thereof, S-alkyl, S-aryl, dialkylamino and N-azolyl groups.

Preferably R^3 is selected from hydroxy, oxyanion and salts thereof, alkyl (such as methyl and substituted methyl) methoxy, ethoxy and substituted ethoxy (such as 2-cyano ethoxy, 2-nitrophenylethoxy and 4-nitrophenylethoxy), allyloxy and substituted allyl, propargyloxy and substituted propargyl, benzyloxy and substituted benzyl, O-9-fluorenylmethyl, propyloxy and substituted propyloxy (such as 1,1,1,3,3,3 hexafluoroisopropyloxy), dimethylamino, diethylamino, methylethylamino, methylisopropylamino, ethylisopropylamino, diisopropylamino, di-n-propylamino, di-n-butylamino, diisobutylamino, dicyclohexylamino, pyrrolidino, piperidino, 2,6-dimethylpiperidino, 2,2,6,6-tetramethylpiperidino, morpholino, 2,6-dimethylmorpholino, hexamethyleneamino, heptamethyleneamino, N-imidazolyl, substituted imidazolyl, N-benzotriazolyl, N-1,2,4-triazolyl, substituted triazolyl, N-tetrazolyl, substituted tetrazolyl, 2-chlorophenoxy, 4-chlorophenoxy, 2-nitrophenoxy, 4-nitrophenoxy, pentaflurophenoxy, 1-benzotriazolyloxy, thiol, thioxyanion and salts thereof, S-t-butyl, S-phenyl, S-2,4-dichlorobenzyl, or S-2,4-dinitrobenzyl.

Preferably R^3 is 2-cyanoethoxy.

D may be a C_1 to C_4 alkylene group or C_3 to C_{12} arylene group. In accordance with the definition of alkylene and arylene groups above, the groups may include one or more heteroatoms and/or heteroatomic groups such as oxygen, sulfur, nitrogen, carboxamido and the like. Preferably D is a methylene or ethylene group.

R^2 may be hydrogen or a thiol protecting group. Preferably, R^2 is selected from hydrogen, alkyl, S-alkylsulfenyl, S-arylsulfenyl, alkylcarboxamidoalkyl, alkoxycarbonyl and acyl groups .

Preferably R^2 is hydrogen, 9-fluorenylmethyl, 2-(2,4-dinitrophenyl)ethyl, t-butyl, 1-adamanthyl, benzyl, substituted benzyl, benzhydryl, triarylmethyl, ethylsulfenyl, t-butylsulfenyl, tritylsulfenyl, 2-nitrobenzenesulfenyl, 2,4-dinitrobenzenesulfenyl, 3-nitro-2-pyridinesulfenyl, acetamidoemethyl, trimethyl acetamidomethyl, benzamidomethyl, benzyloxycarbonyl, acetyl or benzoyl. Most preferably, R^2 is hydrogen, t-butylsulfenyl, trityl or 4-methoxytrityl.

When R^2 is other than hydrogen, the thiol group may be unmasked *in situ* during the coupling reaction with the molecule of formula (I). The in situ deprotection conditions will be readily apparent to the skilled person. For instance, when R^2 is t-butylsulfenyl, the thiol group can be unmasked by reductive cleavage of the disulfide bond using tris-(2-carboxyethyl)phosphine (TCEP) dithiothreitol (DTT) or other suitable reducing agent.

In a further aspect of the present invention there is provided a compound of formula II in which the amino group is protected. Any suitable protecting group R^4 may be used. Preferably, the protecting group R^4 is selected from urethanyl, alkyl, alkylsulfenyl, arylsulfenyl and sulphonyl protection groups.

Preferably R^4 is 9-fluorenylmethoxycarbonyl, allyloxycarbonyl, propargyloxycarbonyl, t-butyloxycarbonyl, benzyloxycarbonyl, 2-(2-nitrophenyl)ethoxycarbonyl, 2-(4-nitrophenyl)ethoxycarbonyl, 2-(2,4-dinitrophenyl)ethoxycarbonyl, heteroarylmethoxycarbonyl, diarylmethyl, triarylmethyl, trityl, 2,6-dioxocyclohexyliden-1-

ylmethyl, substituted 2,6-dioxocyclohexyliden-1-ylmethyl, 2-nitrobenzenesulfenyl, 2,4-dinitrobenzenesulfenyl, 3-nitro-2-pyridinesulfenyl, substituted arenesulfonyl, 2-nitrobenzenesulfonyl, 2,4-dinitrobenzenesulfonyl. Most preferably, R^4 is 9-fluorenylmethoxycarbonyl.

When R^4 is other than hydrogen, the amino group may be unmasked *in situ* during the coupling reaction with the molecule of formula (I). The *in situ* deprotection conditions will be readily apparent to the skilled person. For instance, when R^4 is, 9-fluorenylmethoxycarbonyl (Fmoc), the amino group can be unmasked under mildly basic conditions such as 20% (v/v) pyridine in dimethylformamide.

Either of the compound of formula (I) or (II), but not both, may be attached to a solid support during the coupling reaction. The resultant conjugate may be cleaved from the solid support by methods known in the art.

Alternatively, the coupling of molecules (I) and (II) may be performed in solution. The solution may be either aqueous or non-aqueous, or may comprise a mixture of aqueous and non-aqueous solvents with or without denaturing agent such as urea or guanidinium chloride. The solution may optionally be buffered with a suitable buffer. The solution may optionally comprise, reagent(s) for the transesterification of the thioester in molecule (I), e.g. thiophenol, and/or reagent(s) for the deprotection of the thiol group in molecule (II), e.g. TCEP.

The coupling of molecules (I) and (II) in solution may be optimised by an appropriate choice of solvent, concentration of molecules (I) and (II), and pH. For example, it has been found that the use of acetonitrile or DMF as co-solvent, a high concentration (greater than 0.1 mM, but a lower concentration of 0.001 to 0.1 mM can also be used) of oligonucleotide, and a solution buffered to about pH 6.5 are preferred conditions for coupling oligonucleotides with peptides which are susceptible to a cyclisation side-reaction. A possible undesired cyclisation side-reaction involves attack of a deprotonated N-terminal amido group of the peptide on the thioester carbonyl group. Such a side-

reaction cannot take place in the case of, for example, N-terminal proline or N-terminal N-methyl glycine (sarcosine), since the amido group in each case is secondary and does not contain an acidic hydrogen atom.

According to a further aspect of the present invention there is provided a chemical compound of formula (III) and its use in a method of producing a compound of formula (I) by reaction with M^1-NH_2.

$$R^5\text{-CO-A-CO-S}R^1 \quad \text{(III)}$$

wherein A and R^1 are as previously defined, and

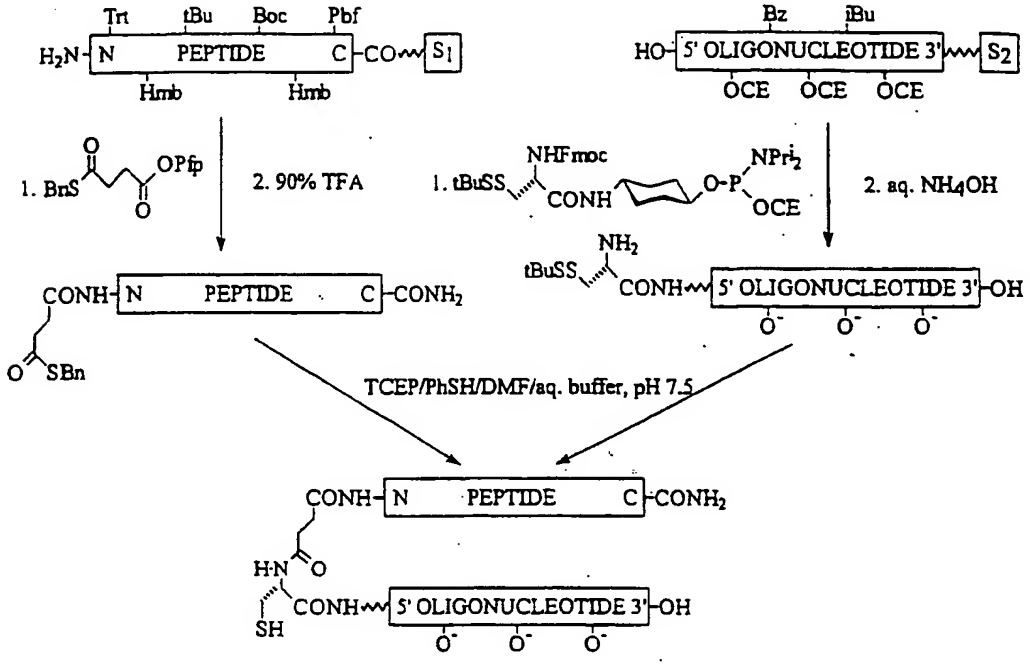
R^5 is selected from hydroxy, oxy anion and salts thereof, alkoxy, aryloxy N-succinimidyloxy, N-(norbornenedicarboximido)oxy, N-benzotriazolyloxy, N-(1,2-dihydro-1-oxo-2,3,4-benzotriazin-2-yl)oxy, halogen and N-azolyl groups; or together with the adjacent CO group forms an anhydride.

Preferably, R^5 is selected from the group consisting of hydroxy, oxy anion and salts thereof, alkoxy (such as 2-cyanoethoxy), substituted or unsubstituted aryloxy (such as 2-nitrophenoxy, 4-nitrophenoxy, 2,4,5-trichlorophenoxy, pentachlorophenoxy, 2,3,5,6-tetrafluorophenoxy and pentafluorophenoxy), substituted or unsubstituted N-(succinimidyl)oxy (such as N-(2-sulfosuccinimidyl)oxy (which is particularly preferred as it improves solubility and maintains reactivity in aqueous and aqueous-organic solutions) and N-(norbornenedicarboximido)oxy, N-(benzotriazolyl)oxy, substituted N-(benzotriazolyl)oxy (such as N-(7-azabenzotriazdyl)oxy, N-(1,2-dihydro-1-oxo-2,3,4-benzotriazin-2-yl)oxy, substituted N-(1,2-dihydro-1-oxo-2,3,4-benzotriazin-2-yl)oxy, halogen (such as fluorine and chlorine), N-azolyl groups (such as N-imidazolyl and substituted imidazolyl, N-benzotriazolyl, N-1,2,4-triazolyl, substituted triazolyl, N-tetrazolyl, substituted tetrazolyl and azido); or together with the adjacent CO group forms a symmetrical anhydride, or a mixed anhydride with carboxylic acids (such as trimethylacetic, 1-adamantane carboxylic or isovaleric), carbonic acid phosphoric or thiophosphoric acids (such as dimethylphosphoric, diethylphosphoric or

diphenylphosphoric acids), phosphonic acids (such as n-propylphosphonic acid), phosphinic or thiophosphinic acids (such as dimethylphosphinic, dimethyl thiophosphinic, diphenylphosphinic or diphenylthiophosphinic acids), sulfonic acids (such as benzene and substituted benzene sulfonic acids, methane sulfonic acid, trifluoromethane-sulfonic acid, trifluoroethane sulfonic acid and polymeric poly (fluorocarbon) sulfonic acid) or hydrocyanic acid.

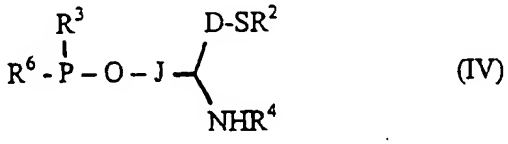
The reaction between a coupling reagent of formula (III) and the amino group of a peptide residue or an amino-modified oligonucleotide residue will chemoselectively form an amide bond between the amino group and the coupling reagent (III) by overall displacement of R^5. Accordingly, R^5 should be chosen such that the adjacent carbonyl is more reactive to amino groups than the carbonyl of the thioester. The choice of R^1 and R^5 to achieve the desired chemoselectivity will be readily apparent the skilled person. The choice of R^5 is also influenced by the intended medium for the reaction with M^1-NH_2. For example when M^1 is a peptide residue selection of R^5 as an N-succinimidyloxy group (such as N-(2-sulfosuccinimidyl)oxy) is particularly preferred as it improves solubility in aqueous-organic mixtures. In non-aqueous media, R^5 is preferably pentafluorophenoxy while R^1 is benzyl.

The preparation and coupling of molecules of general formulae (I) and (II) is exemplified in Reaction Scheme 1. It will be appreciated that preparation and coupling of molecules of general formula (I) and (II) may be carried out by modification of the procedures shown in Reaction Scheme 1 in accordance with conventional synthetic organic chemistry.



Reaction Scheme 1

According to a further aspect of the present invention there is provided a chemical compound of formula (IV) and its use in producing a compound of formula (II) by reaction with M^2-OH.



(IV)

wherein D, J, R^2, R^3 and R^4 are as previously defined; and R^6 is selected from dialkylamino (such as dimethylamino, diethylamino, methylethylamino, methylisopropylamino, ethylisopropylamino, diisopropylamino di-n-propylamino, di-n-

butylamino, diisobutylamino, dicydonexylamino, pyrrolideno, piperidino, 2,6-demethylpiperidino, 2,2,6,6-tetramethyl-piperidino, morpholino and 2,6-dimethylmorpholino); imino (such as hexamethylene imino and heptamethylene imino); halogen (such as fluorine and chlorine); N-azolyl (such as N-imidazolyl and substituted imidazolyl, N-benzotriazoly, N-1,2,4-triazolyl and substituted triazolyl, N-tetrazolyl and substituted N-tetrazolyl); alkoxy (such as 1,1,1,3,3,3-hexafluoroisopropoxy); aryloxy (such as 2-nitrophenoxy, 4-nitrophenoxy, pentafluorophenoxy, 1-benzotriazolyloxy); alkylthio (such as S-tert-butyl) and arylthio (such as S-phenyl). Preferably R^6 is a dialkylamino group, more preferably a diisopropylamino group.

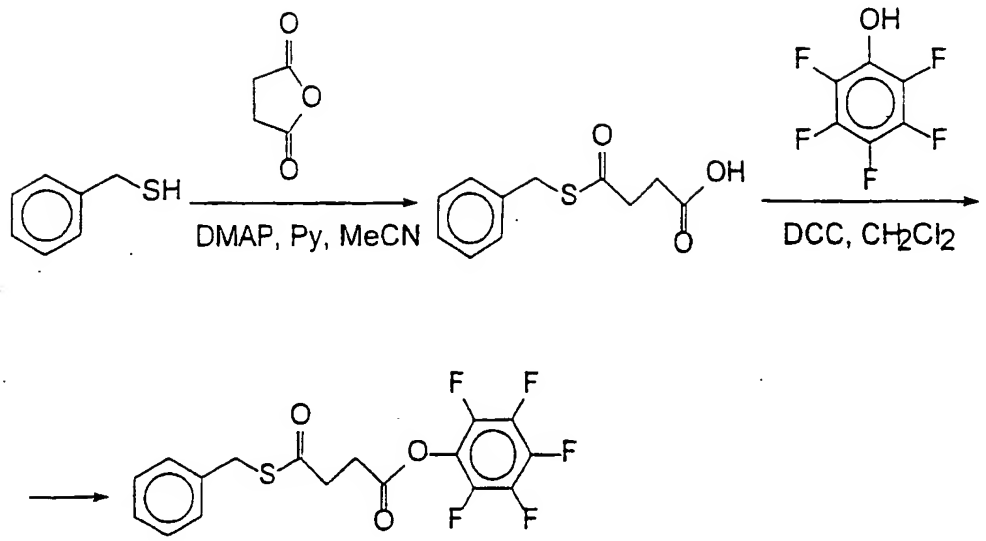
The group represented by R^6 on the coupling reagent (IV) will be displaced by a nucleophilic hydroxy group in M^2-OH (such as an oligonucleotide hydroxy residue) to form the molecule represented by the formula (II). Preferably, the nucleophilic group is the 5'-OH terminus of the oligonucleotide residue.

The coupling reagents (III) and (IV) may be prepared by procedures such as those described in Reaction Schemes 2 and 3. It will be appreciated that coupling reagents of general formulae (III) and (IV) may be prepared by modification of the procedures shown in Reaction Schemes 1 and 2 in accordance with conventional synthetic organic chemistry.

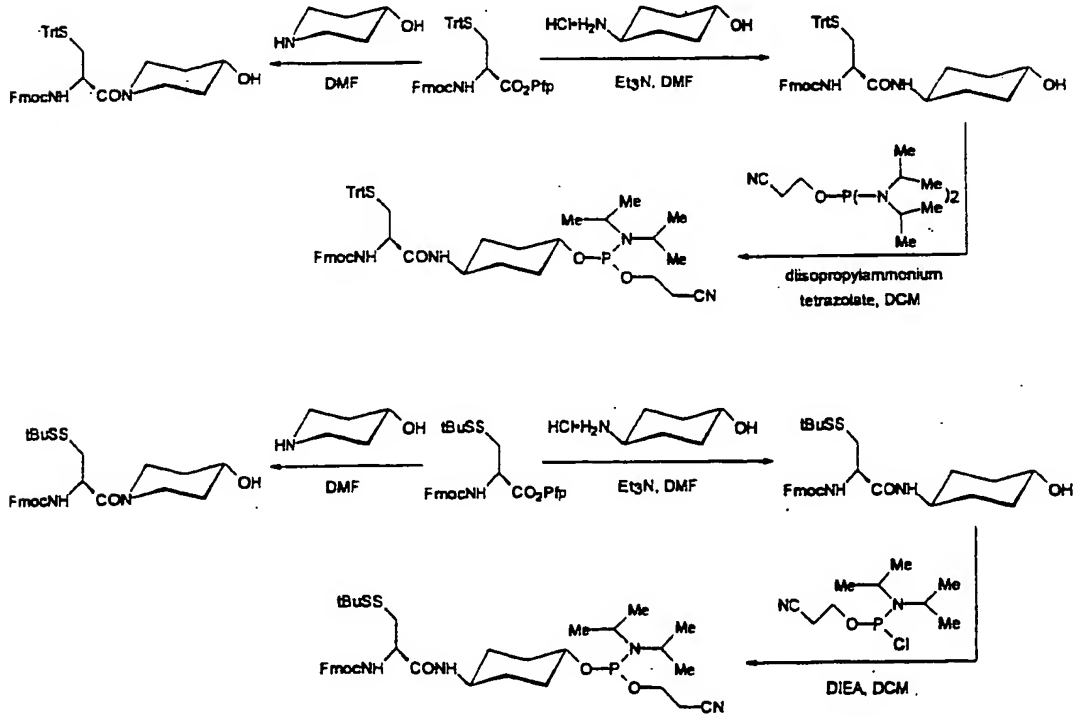
In one aspect of the present invention, the compound of formula (IV) may be used to produce a compound of formula (II) by reaction with a second molecule M^2-OH. The compound of formula (II) is then coupled with a compound of formula (I).

Alternatively, the compound of formula (IV) may be used for modifying molecules of type M^2-OH, which are then coupled with a molecule of type M^1-NH_2 that has been modified by a known route, for example a maleimido group as reported by Eritja *et al* (Tetrahedron, 47 (1991) 4113) and by Soukchareun *et al* (Bioconj. Chem. 9 (1998) 466).

According to a further aspect of the present invention, there is provided a kit comprising coupling reagent (III) and/or (IV). The kit may be used for modifying molecules such as M^1-NH_2 and M^2-OH, which can then be coupled by the native ligation reaction described herein. The kit may further comprise other components which may be used in the coupling reaction. These include one or more components selected from thiophenol, TCEP, urea, guanidinium chloride and sodium phosphate buffer. The kit may also comprise instructions for carrying out the native ligation coupling reaction and/or instructions for modifiying molecules such as M^1-NH_2 and M^2-OH.

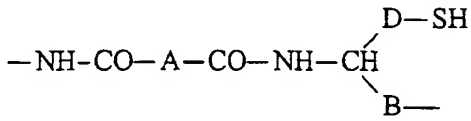


Reaction Scheme 2: Synthesis of coupling reagent (III)



Reaction Scheme 3: Syntheses of coupling reagent (IV)

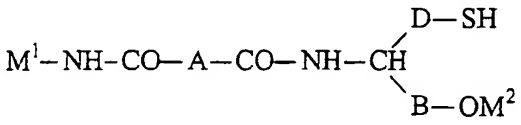
According to the present invention the coupling reaction provides a chemical compound comprising a structural unit of formula (V)



(V)

wherein A, B and D are as defined above.

More particularly, there is provided a chemical compound of the formula (VI)

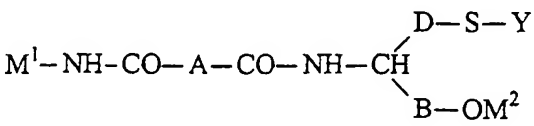


(VI)

wherein M^1, M^2, A, B and D are as defined above.

Where M^1 and M^2 are peptide and oligonucleotide residues respectively, such compounds may have therapeutic utility. For example, the peptide residue may facilitate cellular uptake or intracellular activity of a therapeutic oligonucleotide. The peptide may act as a signal molecule for identification of the location of the oligonucleotide within a cell or for the delivery of the oligonucleotide to a specific cellular location or may enhance the targeting ability of the oligonucleotide towards cellular RNA or DNA in an antisense or triplex therapeutic or diagnostic application.

The thiol group of the compound of formula (VI) is particularly suited to further derivatisation or functionalisation and can, for example, provide a site for attachment of a labelling, reporter or effector group. Accordingly, the invention further provides a chemical compound of the formula (VII)



(VII)

wherein M^1, M^2, A, B and D are as defined above, and Y is a labelling, reporter or effector group.

The invention will now be described in detail with reference to the following examples. It will be appreciated that this invention is described by way of example only and modification of detail may be made without departing from the scope of the invention.

Example 1: Preparation of Coupling Reagent (III)

1). S-Benzyl thiosuccinic acid. Benzyl mercaptane (22 mmol, 2,595 ml) was added under nitrogen to stirred solution of succinic anhydride (20 mmol, 2,0014 g) and 4-dimethylaminopyridine (1 mmol, 122.2 mg) in 25 ml of anhydrous acetonitrile – pyridine (9:1 v/v). Stirring was continued at room temperature for 3 h, evaporated to near dryness, redissolved in sodium bicarbonate solution, pH 8.5, and extracted twice with diethyl ether. Water phase was then cooled at ice bath, acidified with 5N hydrochloric acid to pH 2, white precipitate filtered, washed with ice-cold water and dried in vacuum desiccator over phosphorus pentoxide overnight. Yield of white powder 3.5985 g (80%). ^{1}H-NMR ($CDCl_3$, Ω, ppm): 7.29-7.26 (m, 5H, Ph), 4.16 (s, 2H, CH_2S), 2.94-2.90 (t, 2H, CH_2COS), 2.77-2.72 (t, 2H, CH_2CO_2H).

2). Pentafluorophenyl S-benzyl thiosuccinate. Solution of dicyclohexylcarbodiimide (11 mmol, 2.2696 g) in 15 ml of dichloromethane was added dropwise to stirred and cooled (ice bath) solution of S-benzyl thiosuccinic acid (2.243 g, 10 mmol) and pentafluorophenol (11.5 mmol, 2.117 g) in 25 ml of dichloromethane. Reaction mixture was stirred for 0.5 h on ice bath, then allowed to warm slowly to room temperature, stirred for 4 h and left overnight in a fridge. Dicyclohexylurea precipitate was filtered (2.148 g, 96% yield), solution concentrated in vacuo, redissolved in minimal volume of ethylacetate, filtered again, and hexane was added. After standing overnight in a freezer, crystals were filtered, washed with cold ethylacetate - hexane (1:9 v/v) and dried in vacuo overnight. Yield of

white needles 3.4376 g (88%). After evaporating of mother liquor and further treatment with hexane additional 0.183 g of title compound could be obtained. Total yield of two crops 3.6206 g (92%). ^{1}H-NMR ($CDCl_3$, δ, ppm): 7.30-7.27 (m, 5H, Ph), 4.18 (s, 2H, CH_2S), 3.05 (s, 4H, CH_2CH_2).

Example 2: Preparation of Coupling Reagent (IV)

1). N-Fmoc-S-trityl-L-cysteine 4-hydroxypiperidide. 4-Hydroxypiperidine (2.3 mmol, 232.6 mg) was added with stirring to the solution of N-Fmoc-S-trityl-L-cysteine pentafluorophenyl ester (2 mmol, 1.504 g) in 25 ml of anhydrous acetonitrile, followed by triethylamine (0.5 mmol, 0.07 ml). Reaction mixture was stirred at room temperature for 4 h, until TLC revealed the reaction completed. The mixture was then evaporated to dryness, redissolved in ethylacetate and washed successively with ice-cold 5% wt. citric acid, water, sodium bicarbonate and brine, dried over sodium sulfate, and evaporated to a light brown foam. The residue was chromatographed on silica gel column eluted by 15-30% of acetonitrile in chloroform, appropriate fractions were pooled and evaporated to give 1.3126 g (98%) of the title product as a white foam.

2). N-Fmoc-S-trityl-L-cysteine 4-hydroxy-*trans*-cyclohexylamide. To the slurry of *trans*-4-aminocyclohexanol hydrochloride (2.1 mmol, 318.4 mg) and N-Fmoc- S-trityl-L-cysteine pentafluorophenyl ester (2 mmol, 1.504 g) in 25 ml of anhydrous DMF triethylamine was added (2.2 mmol, 0.307 ml), and resulting solution was stirred at room temperature for 3 h, until TLC revealed reaction completed. Reaction mixture was then evaporated to dryness, redissolved in ethylacetate and washed successively with ice-cold 5% wt. citric acid, water, sodium bicarbonate and brine, dried over sodium sulfate, and evaporated to a light brown foam. The residue was chromatographed on silica gel column eluted by 15-5% hexane in ethylacetate – 0.5% triethylamine, appropriate fractions were pooled and evaporated to give 1.3045 g (95%) of the title product as a white foam.

3). N-Fmoc-S-*tert*-butylsulfenyl-L-cysteine 4-hydroxy-*trans*-cyclohexylamide. To the slurry of *trans*-4-aminocyclohexanol hydrochloride (2 mmol, 303.3 mg) and N-Fmoc-S-*tert*-butylsulfenyl-L-cysteine pentafluorophenyl ester (2 mmol, 1.195 g) and 1-hydroxybenzotriazole (2 mmol, 270.3 mg) in 20 ml of anhydrous DMF, triethylamine was

added (3.1 mmol, 0.446 ml), and resulting solution was stirred at room temperature for 3 h, until TLC revealed reaction completed. Reaction mixture was then evaporated to dryness, white residue transferred to sintered glass filter, washed successively with small amount of DMF, ethanol and diethyl ether, and dried in vacuo. Yield of white powder 0.868 g (82%).

4). N-Fmoc-S-*tert*-butylsulfenyl-L-cysteine 4-hydroxypiperidide. 4-Hydroxypiperidine (2.5 mmol, 253.1 mg) was added with stirring to the solution of N-Fmoc-S-*tert*-butylsulfenyl-L-cysteine pentafluorophenyl ester (2 mmol, 1.195 g) in 20 ml of anhydrous DMF. Reaction mixture was stirred for 3 h at room temperature until TLC revealed completed reaction. The mixture was then evaporated to dryness, redissolved in ethylacetate and washed successively with 5% wt. citric acid, water, sodium bicarbonate and brine, dried over sodium sulfate, and evaporated to a light yellow foam. The residue was chromatographed on silica gel column eluted by 10-0% hexane in ethylacetate, appropriate fractions were pooled and evaporated to give 0.9831 g (95%) of the title product as a white foam.

5). 4-*trans*-N-Fmoc-S-tritvl-L-cysteinylamidocyclohexyl 2-cyanoethyl N,N-diisopropyl phosphoramidite. To a solution of N-Fmoc-S-trityl-L-cysteine 4-hydroxy-*trans*-cyclohexylamide (0.3444 mmol, 0.2352 g) in 10 ml of anhydrous dichloromethane containing 75 mg (1.5 eq) of diisopropylammonium tetrazolate, 2-cyanoethoxy-N,N,N',N'-tetraisopropyl phosphordiamidite (1.15 eq, 0.126 ml) was added, and the mixture was stirred for 6 h at room temperature, until TLC revealed complete conversion. Dichloromethane was then evaporated, residue taken up in ethylacetate, washed with saturated sodium bicarbonate and brine, dried over anhydrous sodium sulfate and evaporated to a small volume. The rest was chromatographed on silica gel column eluted with 25-10% hexane in ethylacetate – 3% triethylamine, appropriate fractions pooled and evaporated to dryness. Yield of a title product 1.3045 g (95%).

6). 4-N-Fmoc-S-trityl-L-cysteinylpiperidyl 2-cyanoethyl N,N-diisopropyl phosphoramidite. To a chilled (ice bath) solution of N-Fmoc-S-trityl-L-cysteine 4-hydroxypiperidide (0.668 g, 1 mmol) in 10 ml of anhydrous dichloromethane containing 3 mmol (0.514 ml) of diisopropylethylamine, 2-cyanoethoxy-N,N-diisopropylaminochlorophosphine (1.3 mmol, 0.29 ml) was added dropwise via syringe ander nitrogen. After 1 h of stirring cold, the mixture was allowed to warm up gradually,

and stirring was continued for 2 h at room temperature. The mixture was then quenched with 0.1 ml of methanol, evaporated to dryness, residue taken up in ethylacetate, washed with saturated sodium bicarbonate and brine, dried over anhydrous sodium sulfate and evaporated to a small volume. The rest was chromatographed on silica gel column eluted with 30-15% hexane in dichloromethane – 3% triethylamine, appropriate fractions pooled and evaporated to dryness. Yield of a title product 0.695 g (80%).

7). 4-*trans*-N-Fmoc-S-*tert*-butylsulfenyl-L-cysteinylamidocyclohexyl 2-cyanoethyl N,N-diisopropyl phosphoramidite. To a chilled (ice bath) solution of 4-*trans*-N-Fmoc-S-*tert*-butylsulfenyl-L-cysteinylamidocyclohexanol (0.834 g, 1.577 mmol) in 15 ml of anhydrous dichloromethane containing 3 eq (0.785 ml) of diisopropylethylamine, 2-cyanoethoxy-N,N-diisopropylamino chlorophosphine (1.5 eq, 0.529 ml) was added dropwise via syringe ander nitrogen. After 1 h of stirring cold, the mixture was allowed to warm up gradually, and stirring was continued for 2 h at room temperature. The mixture was then quenched with 0.1 ml of methanol, evaporated to dryness, residue taken up in ethylacetate, washed with saturated sodium bicarbonate and brine, dried over anhydrous sodium sulfate and evaporated to a small volume. The rest was chromatographed on silica gel column eluted with 25-10% hexane in ethylacetate – 2.5% triethylamine, appropriate fractions pooled and evaporated to dryness. Yield of a title product 0.7567 g (66%).

8) 4-*N*-α-Fmoc-*S*-*tert*-butylsulfenyl-L-cysteinylpiperidyl 2-cyanoethyl *N,N*-diisopropyl phosphoramidite. To a solution of N-α-Fmoc-*S*-*tert*-butylsulfenyl-L-cysteine 4-hydroxypiperidide (1.536 mmol, 0.790 g) in 15 ml of anhydrous CH_2Cl_2 containing 0.329 g (1.920 mmol) of diisopropylammonium tetrazolide, 2-cyanoethoxy-*N,N,N',N'*-tetraisopropyl phosphordiamidite (1.690 mmol, 0.537 ml) was added under N_2 atmosphere, and the mixture was stirred for 3 h at room temperature, until TLC (B) revealed complete reaction. The solvent was then removed by evaporation, the residue was taken up in EtOAc, washed with saturated $NaHCO_3$ solution (x2) and brine, dried over anhydrous Na_2SO_4, filtered through a cotton plug, evaporated to a white foam, re-evaporated several times with CH_2Cl_2, dried *in vacuo* and dissolved in small volume of 10% EtOAc in hexane. The solution was chromatographed on a silica gel column eluted with 10-40% EtOAc in hexane containing 2% triethylamine, appropriate fractions were pooled, evaporated to dryness and rinsed with hexane. The remaining waxy solid was dissolved in

dry CH_2Cl_2, and re-evaporated twice with dry CH_2Cl_2 to give a white foam. Yield of title product 0.455 g (42%). TLC (B): R_f 0.78. ^1H NMR (CD_3CN): δ 1.15-1.19 (m, 12), 1.33 (s, 9), 1.61 (bm, 2), 1.82 (bm, 2), 1.93-1.97 (quintet, 1), 2.16 (s, MeCN), 2.61-2.67 (t, 2), 2.81-2.88 (m, 1), 3.04-3.10 (m, 1), 3.44 (bm, 2), 3.57-3.80 (bm, 6), 4.09 (m, 1), 4.22-4.26 (t, 1), 4.35-4.37 (d, 2), 4.86-4.88 (quadruplet, 1), 6.12-6.17 (t, 1), 7.32-7.37 (t, 2), 7.40-7.45 (t, 2), 7.66-7.68 (d, 2), 7.83-7.85 (d, 2). ^{31}P NMR (CD_3CN): δ 147.10 (61%), 147.03 (19%), 146.94 (20%) (mixture of rotamers). MALDI-TOF MS: (M+H) 715.7 (715.9 calc.), (M+Na) 737.0 (737.9 calc.), (M+K) 753.4 (754.0 calc.).

Example 3: Preparation of Modified Peptide (I)

All peptides were synthesized on the Pioneer™ peptide synthesizer (PE Biosystems) on 0.1 mmol scale, by HATU/DIEA mediated in situ activation protocol supplied by manufacturer. N-α-Fmoc amino acids had standard side chain protection, respectively: 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl on arginine, trityl on asparagine and glutamine, t-butyl on aspartic and glutamic acids, serine, threonine and thyrosine, t-butoxycarbonyl on lysine and tryptophan, and trifluoroacetyl on lysine, were used in 4-fold excess, with no double couplings, and all were of commercial origin. Syntheses were made on either PAL-PEG-PS support (PE Biosystems) or Rink amide NovaGel™ (CN Bioscience). After completion of the desired sequence and N-terminal Fmoc deprotection, the support (0.1 mmol) was transferred to a vial, and then coupling of pentafluorophenyl S-benzylthiosuccinate was performed manually, using 4.5 eq (0.176 g) of the compound and 1 eq (14 mg) of 1-hydroxy-7-azabenzotriazole (HOAt) as a catalyst, in 2 ml of a freshly distilled DMF for 4 h at room temperature. The resin was then washed with 5x4 ml of DMF, 5x3 ml of methanol, and 5x2 ml of diethyl ether, and dried in vacuo. The N-modified peptide was cleaved from the resin and deprotected, using 6 ml of trifluoroacetic acid – benzyl mercaptan – phenol – water (90:5:2.5:2.5 v/v/v/v) cocktail, for 2-6 h at room temperature, depending on arginine content. The resin was filtered, washed with 5x4 ml of TFA, the volume was reduced to cal. 1-2 ml by purging nitrogen, and 40 ml of cold diethyl ether was then added to precipitate the peptide. The slurry was centrifugated, ether decanted, and procedure was repeated three times to remove scavengers. The peptide

pellet was then dried in vacuo, dissolved in 0.1% aqueous TFA/acetonitrile mixture, and subjected to RP HPLC purification on a C_8 column by a gradient of an acetonitrile in 0.1% aqueous TFA. The appropriate fractions were lyophilised and analysed by MALDI-TOF MS, using α-cyano-4-hydroxy cinnamic acid as a matrix. Typical examples are given in **Table 1**.

Table 1. Automated solid phase synthesis of peptide N-terminal S-benzyl thioesters.

Sequence	MALDI-TOF MS	Purity of crude product, % (HPLC)
BnSCO-RQIPK$^{\Psi a}$IWFPNRRK$^{\Psi a}$PFK$^{\Psi a}$K$^{\Psi a}$-$CONH_2$	2714.59	72.0
BnSCO-GRK$^{\Psi a}$K$^{\Psi a}$RRQRRR-$CONH_2$	1791.63	76.5
BnSCO-PTSQSRGDPTGPKE-$CONH_2$	1660.25	88.7
BnSCO-DRVIEVVQGAYRAIRNIPRRIRQG-$CONH_2$	3040.62	28.6
BnSCO-QAKKKKLDK-$CONH_2$	1289.92	90.3
BnSCO-ALPPLERLTL-$CONH_2$	1325.70	74.7
BnSCO-GALFLGFLGAAGSTMGAWSQPKSKRKV-$CONH_2$	2969.05	48.3
BnSCO-Sar-Leu-Gly-Ile-Gly-$CONH_2$	657.91	92.2
BnSCO-PQIK$^{\Psi a}$IWFPNRRK$^{\Psi a}$PFK$^{\Psi a}$K$^{\Psi a}$-$CONH_2$	2715.02	72.0

Example 4: Preparation of Modified Oligonucleotide (II)

Oligonucleotides were synthesized on ABI 380B automated DNA/RNA synthesizer (PE Biosystems) on 1 μmol scale. Standard 3'-nucleoside succinate LCAA-CPG 500A supports were used. After completion of the assembly of the desired sequence and 5'-terminal 4,4'-dimethoxytrityl (Dmt) group cleavage by 2% dichloroacetic acid solution in dichloromethane (v/v), support-bound oligonucleotide was treated on machine with 0.15M solution of modified phosphoramidite reagent (t-butylsulfenyl or trityl S-protected) in anhydrous acetonitrile mixed with 0.5M solution of 1*H*-tetrazole in anhydrous acetonitrile, for 10 min at room temperature. The support was then washed with standard oxidizing solution (0.1M iodine in aqueous pyridine – tetrahydrofuran), washed with acetonitrile and dried. Fmoc amino protecting group can be selectively removed from support-bound oligonucleotide by treating the support with 20% piperidine solution in dimethylformamide (v/v) for 15 min at room temperature. S-Trityl protecting group can be selectively removed on solid support with 0.05M aqueous solution of silver nitrate for 15 min, followed by 0.05M dithiothreitol (DTT) for 5 min (Mag *et al*, Nucl. Acids Res., 19 (1991) 1437). Alternatively, S-Trt protection can be removed in solution with silver nitrate as well (Connolly and Rider, Nucl. Acids Res., 13 (1985) 4485). S-t-Butylsulfenyl group is also sufficiently stable to concentrated aqueous ammonia deprotection at 55°C routinely employed in oligonucleotide synthesis to allow isolation of S-t-butylsulfenyl-protected oligonucleotides, which may be subsequently deprotected by treating with 0.1M aqueous solution of tris-(2-carboxyethyl)phosphine, pH 7.0, at room temperature overnight. Both S-trityl and S-t-butylsulfenyl provide convenient hydrophobic handles for reverse phase purification of synthesized oligonucleotides. Routinely, S-protected cysteine-modified oligonucleotides were isolated after RP HPLC on μBondapak C_{18} column eluted by a gradient of an acetonitrile in 0.1M aqueous ammonium or triethylammonium acetate solution, pH 7.0, desalted on Sephadex G-10 or G-25 column, lyophilised and analysed by MALDI-TOF MS using 2,6-dihydroxyacetophenone – ammonium citrate as matrix. Typical examples are given in **Table 2.**

Table 2. Automated solid phase synthesis of 5'-cysteinyl oligonucleotides.

Sequence	MALDI-TOF MS	Purity of crude product, % (HPLC)
Cys(SBu^t)-TTT TT	1825.13	87.1
Cys(Trt)-TTT TT	1983.16	76.6
Cys-TTT TT	1738.24	82.2
Cys(SBu^t)-CTC CCA GGC TCA AAT	4874.42	82.6
Cys(SBu^t)-GCT CCC AGG CTC AAA	4888.15	89.7
Cys(Trt)-CTC CCA GGC TCA AAT	5017.05	91.2
Cys(Trt)-GCT CCC AGG CTC AAA	5043.74	89.2
Cys(Trt)-AGC TCC CAG GCT CAA	5043.88	90.5
Cys(SBu^t)-AGC TCC CAG GCT CAA	4887.98	86.5
Cys-GCT CCC AGG CTC AAA	4803.15	100

Example 5: Coupling Reactions (Solution phase)

The solution phase peptide-oligonucleotide coupling reactions were performed using four different experimental procedures (Methods A, B, C and D). Typical examples are given in **Table 3**.

Table 3. Synthesis of Peptide-N-to-5'-oligonucleotide conjugates

Oligonucleotide sequence	Peptide Sequence	Method	MALDI-TOF MS	Yield, %
TTT TT	PTSQSRGDPTGPPKE	A	3276.72	75
GCT CCC AGG CTC AAA	PTSQSRGDPTGPPKE	B	6340.17	65
GCT CCC AGG CTC AAA	Sar-Leu-Gly-Ile-Gly	C	5349.05	26
GCT CCC AGG CTC AAA	ALPPLERLTL	C	6043.43	22
AGC TCC CAG GCT CAA	Sar-Leu-Gly-Ile-Gly	D	5348.45	43
AGC TCC CAG GCT CAA	ALPPLERLTL	D	6043.88	51

Example 5a (Method A). 0.9 μmol of crude oligodeoxynucleotide TTT TT, 5'-modified with 4-(S-*tert*-butylsulfenyl)cysteinylamido-*trans*-cyclohexyl phosphate, were dissolved in 1 ml of 25% v/v N,N-dimethylformamide – 0.1M sodium phosphate buffer, pH 7.5, containing 7M urea and 0.1M *tris*-(2-carboxyethyl)phosphine, and 4.5 μmol of lyophilised

peptide PTSQSRGDPTGPKE amide, N-terminally modified with S-benzyl thiosuccinyl moiety, were added followed by 4% v/v thiophenol. The mixture was incubated at ambient temperature for 24 h, and then analysed by RP-HPLC (μBondapak C_{18} analytical column, detection at 218 and 254 nm, flow rate 1 ml/min, buffer A: 0.1M ammonium acetate, pH 7.0, buffer B: acetonitrile, gradient: 5 min 2% B, 20 min 40% B, 25 min 100% B, retention time: starting S-*tert*-butyl oligonucleotide 16.608 min, reduced oligonucleotide 14.575 min, conjugate 15.258 min). After isolation by preparative HPLC, conjugate was assessed by MALDI-TOF MS: calculated 3277.59, observed 3376.72. Yield: 75% calculated on starting oligonucleotide.

Example 5b (Method B). 0.6 μmol of crude oligodeoxynucleotide GCTCCCAGGCTCAAA, 5'-modified with 4-(S-*tert*-butylsulfenyl)cysteinylamido-*trans*-cyclohexyl phosphate, were dissolved in 1 ml of 25% v/v N,N-dimethylformamide – 0.1M sodium phosphate buffer, pH 7.5, containing 0.1M *tris*-(2-carboxy ethyl)phosphine, and 3 μmol of lyophilised peptide PTSQSRGDPTGPKE amide, N-terminally modified with S-benzyl thiosuccinyl moiety, were added followed by 4% v/v thiophenol. The mixture was incubated at ambient temperature for 24 h, and then analysed by RP-HPLC (μBondapak C_{18} analytical column, detection at 218 and 254 nm, flow rate 1 ml/min, buffer A: 0.1M triethylammonium acetate, pH 7.0, buffer B: acetonitrile, gradient: 5 min 2% B, 20 min 40% B, 25 min 100% B, retention time: starting S-*tert*-butyl oligonucleotide 16.875 min, reduced oligonucleotide 16.083 min, disulfide oligonucleotide 24.750 min, conjugate 15.541 min, disulfide conjugate 26.933 min). After isolation by preparative HPLC, conjugate was assessed by MALDI-TOF MS: calculated 6340.51, observed 6340.17. Yield: 65% calculated on starting oligonucleotide.

Example 5c (Method C) The coupling reactions were performed under the following conditions: 0.05 mM modified oligonucleotide, 10 equiv. modified peptide, 0.1 M TCEP, titrated to pH 6.5 by addition of 20% sodium hydroxide solution, 2% PhSH (v/v), 25% acetonitrile, 48 h, room temperature.

Example 5d (Method D) The coupling reactions were performed under the following conditions: 0.01 mM modified oligonucleotide, pretreated with 0.2 M TCEP, pH 6.5, for 3 h at room temperature, then 10 equiv. modified peptide in an equal volume of 50%

aqueous DMF was added together with PhSH (2% v/v final) and kept at room temperature for 48 h.

Example 6: Coupling reaction (solid phase)

Solid phase oligonucleotide synthesis column with 1 μmol of crude oligodeoxynucleotide CTCCCAGGCTCAAAT, 5'-modified with 4-(N-Fmoc-S-*tert*-butylsulfenyl)cysteinylamido-*trans*-cyclohexyl 2-cyanoethyl phosphate, still attached to the support, was treated in syringe first with 20% piperidine in N,N-dimethylformamide (v/v) for 15 min, washed with 5 ml DMF, then with 1 ml of 0.5M solution of dithiothreitol in N,N-dimethylformamide – water (1:1 v/v) for 2 h, and washed with 10 ml DMF – water (1:1 v/v). Then 1 ml of solution of 5 μmol of lyophilised peptide GRK^{Tfa}K^{Tfa}RRQRRR amide (Tfa – trifluoroacetyl), N-terminally modified with S-benzyl thiosuccinyl moiety, in 25% DMF – 0.1M sodium phosphate buffer, pH 7.5, containing 4% v/v thiophenol, was added via syringe. The column was incubated at ambient temperature for 24 h, and then washed with 10 ml of 25% acetonitrile – water (v/v). Then 1 ml of 0.5M iodoacetamide solution in 40% DMF – 0.1M sodium phosphate buffer, pH 7.5, was added via syringe, and the column incubated at ambient temperature for further 24 h. After washing with 10 ml of DMF – water (1:1 v/v) support was dried, transferred to screw-capped vial and treated with 25% aqueous ammonia solution at 55°C for 16 h. The glass beads were decanted, washed with 0.5 ml of 25% aqueous ammonia and 0.5 ml of water, the volume of supernatant was reduced to 250 μl, and the mixture was then analysed by RP-HPLC (μBondapak C_{18} analytical column, detection at 218 and 254 nm, flow rate 1 ml/min, buffer A: 0.1M ammonium acetate, pH 7.0, buffer B: acetonitrile, gradient: 5 min 2% B, 20 min 40% B, 25 min 100% B, retention time: starting S-*tert*-butyl oligonucleotide 15.025 min, reduced oligonucleotide 13.338 min, conjugate 14.016 min). Yield: cal. 45% by HPLC.

Example 7: Alternative Coupling Reaction

A 3'-fluoresceinyl 15-mer oligodeoxyribonucleotide dCTCCCAGGCTCAAAT was synthesised on a 1 μmole scale using a commercially available fluorescein-derivatised

controlled poor glass support. Then the cysteinylpiperidine phosphoramidite reagent (t-butylsulfenyl protected) was coupled and, after standard iodine oxidation, the terminal Fmoc group was removed by brief treatment with piperidine. Following standard aqueous ammonia deprotection and release into solution, the resultant 5'-*S-tert*-butylthiocysteine 3'-fluorescein oligonucleotide derivative showed by reversed phase HPLC a single main peak at 18.3 minutes elution time. After HPLC purification, the yield was 20.7 A_{260} units. The product showed a single peak by HPLC and a single band by polyacrylamide gel electrophoresis. The MALDI-TOF mass spectra showed a mass of 5424 Da (calculated 5422). When this oligonucleotide was treated with 0.2 M TCEP solution (pH 6.5) for 30 min at room temperature, the free thiol was obtained quantitatively as observed by HPLC (elution time 16.7 min). The conjugation reaction of this 5'-cysteine-3'-fluorescein oligodeoxynucleotide with maleimide peptides in aqueous solution by methods similar to those previously reported (Soukchareun *et al*, Bioconj. Chemistry, 9, 1998, 466-475) showed that the oligonucleotide was in each case completely consumed within 16 h.

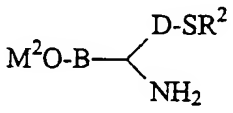
CLAIMS

1. A method of linking a first molecule M^1-NH_2 with a second molecule M^2-OH comprising reaction of a compound of formula (I)

M^1-NH-CO-A-CO-SR^1 (I)

wherein M^1 is the residue of a molecule bearing an amino group
A is an alkylene or arylene group,
R^1 is alkyl or aryl,

with a compound of formula (II)



(II)

M^2 is the residue of a molecule bearing a hydroxy group
wherein B is a linker
D is a C_{1-4} alkylene group or C_{3-12} arylene group
R^2 is hydrogen or a thiol protecting group.

2. A chemical compound of formula (I) as defined in claim 1.

3. A chemical compound according to claim 2 wherein M^1 comprises a peptide residue.

4. A chemical compound according to claim 2 or 3 wherein A comprises a C_{1-4} alkylene group.

5. A chemical compound according to claim 4 wherein A comprises an ethylene (-CH_2-CH_2-) or n-propylene (-CH_2-CH_2-CH_2-) group.

6. A chemical compound according to any one of claims 2 to 5 wherein R^1 comprises a C_{1-18} alkyl or C_{3-10} aryl group.

7. A chemical compound according to claim 6 wherein R^1 is selected from the group consisting of t-butyl, substituted or unsubstituted benzyl, substituted or unsubstituted phenyl, 2-pyridyl, 4-pyridyl, cyanomethyl carboxamidomethyl, 2-carboxamidoethyl and trifluoroethyl.

8. A chemical compound of formula (II) as defined in claim 1.

9. A chemical compound according to claim 8 wherein M^2 comprises an oligonucleotide residue.

10. A chemical compound according to claim 8 or 9 wherein the linker B comprises a group of the formula

-X-J-

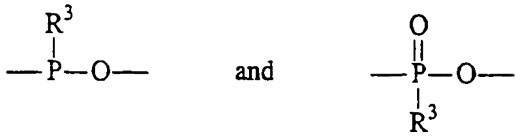
wherein J is an alkylene or arylene group
X is the residue of a functional group capable of reacting with a hydroxy group.

11. A chemical compound according to claim 10 wherein J is a C_{1-18} alkylene group or C_{3-12} arylene group.

12. A chemical compound according to claim 11 wherein J comprises a moiety derived from trans-4-aminocyclohexanol or 4-hydroxypiperidine.

13. A chemical compound according to any one of claims 10 to 12 wherein X is a phosphate, thiophosphate, phosphonate or phosphite residue.

14. A chemical compound according to claim 13 wherein X comprises a group selected from



wherein R^3 is selected from hydroxy, oxy anion and salts thereof, alkyl, alkoxy, aryloxy, thiol, thioxy anion and salts thereof, S-alkyl, S-aryl, N-azolyl, dialkyl amino groups.

15. A chemical compound according to claim 14 wherein R^3 is a 2-cyanoethoxy group.

16. A chemical compound according to any one of claims 8 to 15 wherein D is a methylene or ethylene group.

17. A chemical compound according to any one of claims 8 to 16 wherein R^2 is selected from hydrogen, alkyl, S-alkylsulfenyl, S-arylsulfenyl, alkylcarboxamidoalkyl, urethanyl and acyl groups.

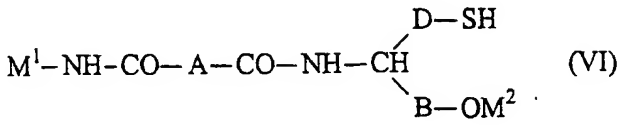
18. A chemical compound according to claim 17 wherein R^2 is hydrogen tert-butyl sulfenyl or trityl.

19. A chemical compound according to any one of claims 8 to 18 wherein the amino group of the compound of formula II is protected.

20. A chemical compound according to claim 20 wherein the amino group is protected with a protecting group R^4 selected from urethanyl, alkyl, alkylsulfenyl, aryl sulfenyl and sulfonyl protecting groups.

21. A chemical compound of the formula (III)

27. A chemical compound according to claim 26 of the formula (VI)



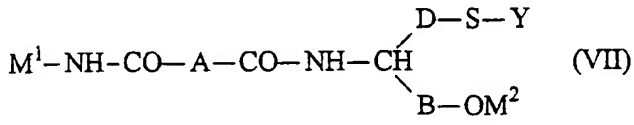
(VI)

wherein M^1, M^2, A, B and D are as defined in any preceding claim.

28. A chemical compound according to any one of claims 2 to 27 wherein the compound is linked to a solid support.

29. A method according to claim 1 wherein M^1, M^2, A, B, D, R^1 and R^2 are as defined in any of claims 4 to 23.

30. A chemical compound of the formula (VII)



(VII)

wherein M^1, M^2, A, B and D are as defined in any preceding claim
Y is labelling, reporter or effector group.

31. Use of a chemical compound according to any one of claims 2 to 28 in a process for linking a peptide and an oligonucleotide.

32. A chemical compound according to claim 27 or 30 for use in therapy.

33. A method of producing a compound of formula (I) comprising reaction of a compound of the formula

M^1-NH_2

with a chemical compound according to claim 21 or 22 wherein M^1, A, R^1 and R^5 are as defined in any preceding claim.

34. A method of producing a compound of formula II comprising reaction of a compound of the formula

M^2-OH

with a chemical compound according to any one of claims 23 to 25 wherein M^2, B, D, R^2, R^3, R^4 and R^6 are as defined in any preceding claim.

35. A kit comprising a chemical compound according to claim 21 or 22 and/or a chemical compound according to any one of claims 23 to 25.